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ORIGINAL PAPER

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# Sensitivity of a polymerase chain reaction-based assay to detect *Onchocerca volvulus* DNA in skin biopsies

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**Abstract** A polymerase chain reaction (PCR) of a 150-bp tandem repeat of *Onchocerca volvulus* (O-150) combined with Southern-blot hybridization to species-specific DNA probes was employed for DNA detection. O-150 was amplified from parasites originating from Uganda, Benin, Cameroon, Liberia, Ghana, Burkina Faso, Mali, and Zaire and was successfully hybridized to digoxigenin-labeled oligonucleotides. To investigate the sensitivity of the PCR, 2 skin biopsies were taken from each of 227 persons from Uganda with proven *O. volvulus* infections but with low microfilaria (mf) densities due to ivermectin treatment. One biopsy was tested by PCR and the other was digested using collagenase to assess the total number of mf. The PCR revealed 76.2% of the samples to be positive, and the collagenase method showed that 78.9% were positive, indicating similar sensitivity for the two methods. It is probable that for both techniques the biopsy must contain at least one live mf or fragments of a dead mf. In this study, no free or circulating *O. volvulus* DNA could be detected in skin biopsies by PCR.

## Introduction

In recent years, several molecular biology methods have been developed for the diagnosis and characterization of human onchocerciasis. As in many other infectious diseases, the polymerase chain reaction (PCR) has been successfully employed for the diagnosis of onchocerciasis in humans (Zimmerman et al. 1994). In addition, the application of PCR for strain differentiation of *Onchocerca volvulus* and species identification from infected vectors has been described (Meredith et al. 1991). All these procedures are based on the amplification and DNA probing of a variable tandemly repeated DNA sequence family with a unit length of 150 bp (O-150) and more than 4000 copies in the haploid genome (Meredith et al. 1989). The analysis of this repeat revealed characteristics allowing the separation of *O. volvulus* from other *Onchocerca* species (Harnett et al. 1989; Meredith et al. 1989; Zimmerman et al. 1993).

There is a need to develop a sensitive means of diagnosis of patients with low microfilaria (mf) densities, particularly after microfilaricidal treatment or in patients with sowda, as well as to detect mf-negative putatively immune persons living in endemic areas. It has been reported that a PCR-based assay on skin biopsies is more sensitive than parasitological methods and overcomes many of their deficiencies for the diagnosis of active onchocerciasis in humans (Zimmerman et al. 1994). This method has been employed on skin biopsies to identify truly infection-free persons (Elson et al. 1994; Freedman et al. 1994), but the results have depended on the reference method used. It has been speculated that persons without mf harbor *O. volvulus* DNA in their skin that can be detected by PCR (Freedman et al. 1994). The results of these studies prompted the suggestion that further research be done to investigate whether a positive PCR signal in patients with no emerging mf in their skin snips is due to free DNA in the biopsy or to nonmotile mf (WHO 1993). The total number of mf in skin snips, including nonmotile mf and fragments of mf, can be assessed by collagenase digestion of skin snips (Schulz-Key 1978). Hence, the collagenase digestion method was used to detect mf in skin biopsies and to evaluate the mf density.

The present study was performed to elucidate if free *O. volvulus* DNA occurs in skin snips of infected persons without mf and to evaluate the sensitivity of the PCR. Infected persons who had very few mf in their skin, if any, were examined using the PCR. Subsequently, the amplification products were further characterized with nonra-

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dioactive, digoxygenin (DIG)-labeled *O. volvulus*-specific DNA probes.

## Materials and methods

### Collection of samples and collagenase digestion

Skin biopsies were collected from persons living in Kigoyera parish, western Uganda. In this region, *Simulium neavei* is the vector of *Onchocerca volvulus*. The area was studied intensively in 1991 (Fischer et al. 1993) before mass treatment with ivermectin was started. The last ivermectin treatment with a single dose of 150 µg/kg body weight was carried out in January or July of 1993. Between November and December of 1993, two skin snips were taken from the buttocks using a Holth or modified Walser punch. The average weight of the biopsies was 4 mg. One biopsy was immediately preserved in 80% ethanol and stored in a refrigerator for PCR testing. The other biopsy was weighed, placed in isotonic salt solution in a microtitration plate, and incubated overnight. The solution was then pipetted onto a slide for microscopic examination using a 63-fold magnification. The remaining biopsy was then digested with 0.5% collagenase (Boehringer, Mannheim, Germany) according to Schulz-Key (1978), and the digested material was examined microscopically to detect the mf that had not emerged after incubation in saline. In addition, an mf-negative control group of 19 persons, who showed no mf in skin snips either before ivermectin treatment or at reexamination, was selected.

To check the suitability of the chosen primers, approximately 1 ng genomic DNA extracted from several pools of female *O. volvulus* was subjected to PCR. The parasites were preserved in liquid nitrogen or in ethanol. They originated from western Uganda (Kigoyera, 0°52′N and 30°32′E), Benin (Aplahoue, 6°57′N and 2°12′E, forest area), Cameroon (Basia, Sangana river, forest area), Liberia (Mauwa, 6°52′N and 10°20′W, forest area), two foci in Ghana (Ahamansu, 8°43′N and 0°33′E, forest area; Wiasi, 10°21′N and 1°20′W, savanna area; from the latter, only mf in skin biopsies were available), Burkina Faso (Hemkoa, 10°41′N and 3°1′W, savanna area), Mali (Manambougou, Missira, Foura, savanna area), and Zaire (Bas, forested area with a high level of blinding onchocerciasis; the savanna strain according to Meredith et al. 1991). For comparison, DNA samples extracted from the cattle parasites *O. ochengi, O. dukei,* and *O. armillata* from northern Cameroon as well as *O. gutturosa* from western Uganda were examined by PCR.

### DNA extraction

The preserved skin snip was incubated at 56° C for 1 h in a solution of 25 µl nucleolysis buffer [0.05 *M* NaCl, 0.01 *M* ethylenediaminetetraacetic acid (EDTA), 0.05 *M* TRIS base], 2.5 µl 10% sodium dodecyl sulfate (SDS), and 0.25 µl 1 *M* dithiothreitol (DTT). Afterward 1 µl proteinase K (20 mg/ml) and 100 µl nucleolysis buffer were added to dilute the DTT and the solution was incubated for at least 2 h at 56° C. A phenol/chloroform extraction was then performed and the DNA was ethanol-precipitated overnight at –20° C. The DNA was pelleted by centrifugation at 13000 rpm for 20 min, dried, and resuspended in 50 µl TRIS-EDTA buffer (0.5 *M* TRIS base, 0.05 *M* EDTA). In all, 3 µl of each extract was subjected to PCR. During the DNA preparation and the PCR, standard precautions were applied to prevent any contamination. DNA solutions prepared from biopsies from persons without *O. volvulus* infection or TE buffer were used as negative controls, since there was no difference between their PCR results. Unless specifically noted, all chemicals were obtained from Sigma-Aldrich (Deisenhofen, Germany) or Merck (Darmstadt, Germany). All oligonucleotides used in this study were customer syntheses of Biometra (Göttingen, Germany).

### Polymerase chain reaction

PCR amplifications were performed employing the Prime Zyme Polymerase Kit (Biometra), which included a thermostable DNA polymerase from *Thermus brockianus* with a half-life of 2.5 h at 96° C and a 2-fold lower error frequency than that of Taq DNA polymerase according to the specifications of the manufacturer. The PCR mix contained 1×PCR buffer (10 m*M* TRIS-HCl, pH 8.8, 50 m*M* KCl, 0.1% Triton X-100), 4 m*M* $MgCl_2$, 100 µ*M* desoxynucleotide triphosphates (dNTPs), 4 µ*M* primer S3 and primer S4, and 1 U polymerase/50 µl. The sequences of primers S3 and S4 were 5′ATCAATTTTGCAAAATGCG3′ and 5′AATAACTGATGACCTATGACC3′, respectively. The thermocycling program started with 5 min of denaturation at 95° C followed by 34 cycles of denaturation for 1 min at 90° C, annealing for 1 min at 60° C, and extension for 2 min at 72° C (Uno Thermocycler, Biometra). Of the PCR product of each sample, 13 µl was separated on a 2% agarose gel using TAE electrophoresis buffer and was visualized with ethidium bromide/UV illumination.

### Southern blotting and DNA hybridization

The amplified DNA was denatured, neutralized, blotted onto a nylon membrane (Nylonbind A, 0.045 µm; Serva, Heidelberg, Germany) using a vacuum blotter (Vacu-Blot, Biometra), and then fixed by baking for 2 h at 80° C. The blots were prehybridized for at least 1 h in hybridization fluid containing 15 ml formamide, 6 ml blocking solution (DIG detection kit, Boehringer), 7.5 ml 20×SSC, 0.3 ml laurylsarcosine (100 mg/ml), 60 µl 10% SDS solution, and 1.14 ml sterile water. The blots were then hybridized in at least 2.5 ml hybridization fluid per 100-$cm^3$ membrane containing 0.05 nmol of the DNA probe/ml hybridization fluid. As *O. volvulus*-specific DNA probes the DIG-labeled oligonucleotides S9 (Meredith et al. 1991), with the sequence 5′(Di)AAATTGATTATTAACAGATGACCTATGACATATAA3′, and OvS2 (Zimmerman et al. 1993), with the sequence 5′(Di)AATCTCAAAAAACGGGTACATAC3′, were used. The blots were hybridized overnight in a hybridization oven (Compact Line OV4, Biometra) at 50° C (S9) or at 42° C (OvS2). Stringent washes were performed for 2×5 min in 2×SSC, 0.1% SDS and for 2×5 min in 0.1×SSC, 0.1% SDS at 50° C. The DIG detection was performed using a commercial DIG detection kit (Boehringer) according to the protocol of the manufacturer.

### Evaluation of sensitivity

Only samples in which PCR products hybridized to either the S9 or the OvS2 probe were classified as PCR-positive. The sensitivity of using either one or two skin snips examined by the two different methods was calculated according to Taylor et al. (1987) as follows:

$$\frac{\text{Number who would be detected as being positive by that test} \times 100}{\text{Number of true positives}}.$$

Individuals were considered true positives (i.e., infected with *O. volvulus*) if they had been mf-positive in a previous examination before ivermectin mass treatment in their village had started.

## Results

### Detection of *Onchocerca volvulus* DNA

To determine the applicability of the described PCR-based assay for the detection of *O. volvulus* DNA from different geographical regions of Africa, genomic DNA extracted from female *O. volvulus* from various African

**Table 1** Applicability of the described PCR-based assay to detect *Onchocerca volvulus* DNA in specimens from various African countries. For comparison, *Onchocerca* species of African cattle were included in the presentation

| Species, origin | Strain | S3/S4 primer | Hybridization with | |
|---|---|---|---|---|
| | | | S9 probe | OvS2 probe |
| *O. volvulus:* | | | | |
| Uganda | *Simulium neavei*-transmitted | + | + | + |
| Benin | Forest | + | + | + |
| Cameroon | Forest | + | + | + |
| Liberia | Forest | + | + | + |
| Ghana | Forest | + | + | + |
| Ghana | Savanna | + | + | + |
| Burkina Faso | Savanna | + | + | + |
| Mali | Savanna | + | + | + |
| Zaire | Savanna | + | + | + |
| *O. ochengi:* | | | | |
| Cameroon | "Cattle" | + | – | – |
| *O. dukei:* | | | | |
| Cameroon | "Cattle" | – | – | – |
| *O. armillata:* | | | | |
| Cameroon | "Cattle" | – | – | – |
| *O. gutturosa:* | | | | |
| Uganda | "Cattle" | – | – | – |

countries was tested. All samples examined, regardless of their strain, and *O. ochengi* were successfully amplified by PCR. DNA of *O. gutturosa, O. armillata,* and *O. dukei* was not amplified. For the specification of the amplification product it was transferred onto a nylon membrane and hybridized to the two different DIG-labeled oligonucleotide DNA probes S9 and OvS2. All *O. volvulus* samples examined hybridized well with these DNA probes, whereas *O. ochengi, O. gutturosa, O. armillata,* and *O. dukei* did not (Table 1).

For the detection of *O. volvulus* DNA in the skin, biopsies of onchocerciasis patients from western Uganda were collected and their DNA was extracted and subjected to PCR. The described PCR assay revealed no obvious correlation between the amount of amplified DNA and the mf density in the skin snip (Fig. 1A). Sometimes the bands of the PCR products were not easy to detect using an ethidium bromide-stained agarose gel; therefore, for characterization of the amplification product, Southern blots were hybridized to the DIG-labeled DNA probe S9 (Fig. 1B). Samples from a total of 227 persons were tested with the PCR. An amplification product was clearly visible in 153 (67.4%) samples, but 173 (76.2%) of all samples hybridized with the specific DNA probe. Since the *O. volvulus*-specific DNA probe S9 overlaps with the oligonucleotides used to prime the PCR, 110 samples, of which 83 samples were amplified and hybridized to S9, were randomly selected from the 227 samples and also hybridized to the species-specific probe OvS2 (Fig. 1C). All these 83 samples hybridized at the chosen stringencies with the OvS2 probe as well. However, less background was observed when the blot was probed with the oligonucleotide S9 because it has a length of 35 nucleotides, whereas the probe OvS2 has only 22 nucleotides. The DNA probe S9 generally reacted with amplification products with a length of 300 bp and longer. The domain to which the probe S9 binds is located at the end of O-150, and the full length of this domain is represented in only a dimer or multimer of the repeat. In contrast, the OvS2 probe also reacted with PCR products of 150-bp length since it binds in the center of O-150 (Fig. 1B, C).

## Comparison of PCR and parasitological examination

For evaluation of the sensitivity of the PCR applied to skin biopsies, only persons identified as mf carriers during the baseline survey in 1991, before the initiation of mass treatement with ivermectin, were analyzed. The geometric mean of the mf density in the 227 persons examined was 10.4 mf/mg in 1991 and 3.4 mf/mg skin in 1993, at the time of the present study. During the same period the prevalence of mf carriers in this group declined from 100% to 83%.

After the incubation of 227 skin biopsies in isotonic saline solution, 168 were mf-positive, revealing a sensitivity of 74%. In comparison with the PCR-based assay there was no significant difference in sensitivity (chi-square test, $P$=0.91). The PCR assay of the corresponding skin snip showed a sensitivity of 76.2% (Table 2). The highest sensitivity of 78.9% was obtained using the collagenase technique (Table 3), but, again, there was no significant difference from the result of the PCR assay (chi-square test, $P$=0.88). By the collagenase digestion method, fragments and degenerating mf (Fig. 2) were detected as well, and there were also single biopsies containing only these nonemerging mf.

Of the 227 samples, 16 (7.1%) were PCR-negative in the first biopsy and mf-positive as determined by collagenase digestion of the second biopsy. This number was not significantly different from the 10 (4.4%) biopsies

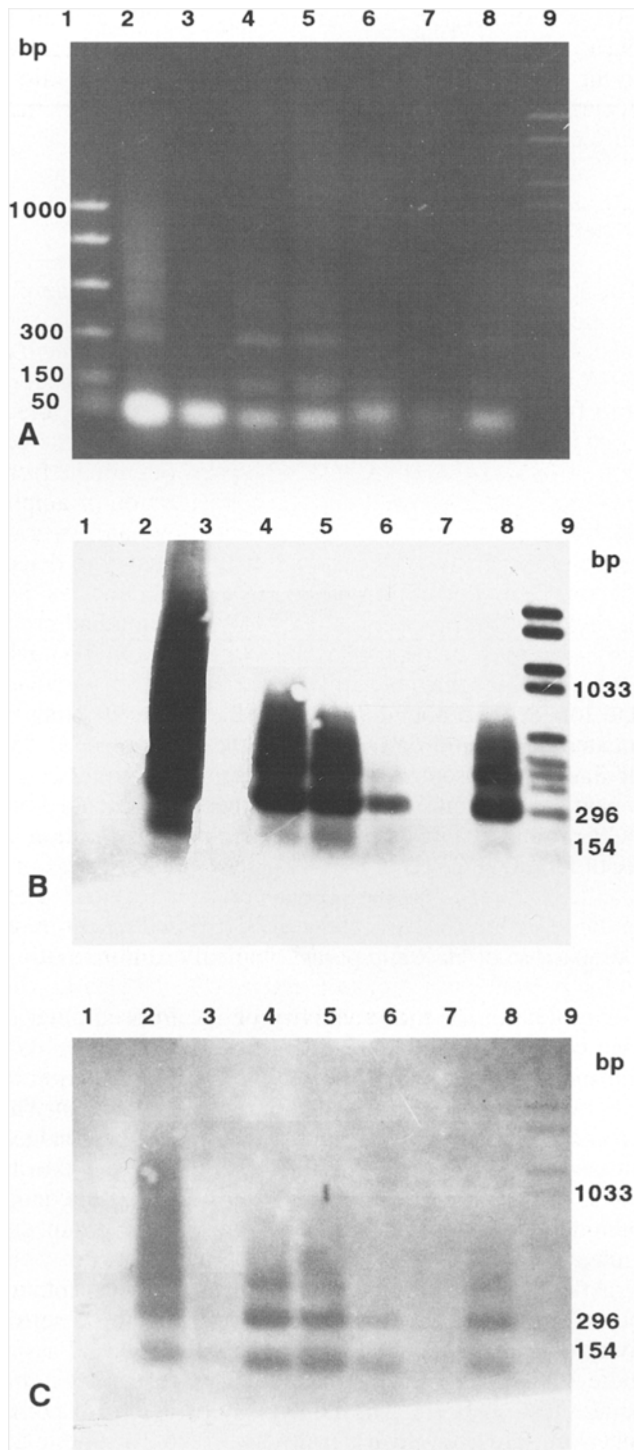


**Fig. 1A–C** Amplification and species-specific DNA probing to detect *Onchocerca volvulus* in skin biopsies of persons from Uganda. **A** Ethidium bromide-stained 2% agarose gel showing amplification products. Mf densities were evaluated by processing of a second skin biopsy with collagenase digestion (*Lane 1* length standard, *lane 2* positive control – adult *O. volvulus* from Uganda, *lane 3* negative control – TE buffer, *lane 4* biopsy mf-negative but PCR-positive, *lane 5* biopsy with a density of 1.9 mf/mg and PCR-positive, *lane 6* biopsy with a density of 78.7 mf/mg but with weak bands in the PCR, *lane 7* biopsy with a density of 1.3 mf/mg but PCR-negative, *lane 8* biopsy with three nonmotile mf and PCR-positive, *lane 9* DIG-labeled length standard). **B** Southern blot of the gel described in *A* hybridized with the DIG-labeled oligonucleotide DNA probe S9, which is specific for *O. volvulus* and binds to PCR products of 300-bp length and longer. **C** Southern blot of the gel described in *A* hybridized with the DIG-labeled oligonucleotide DNA probe OvS2, which is specific for *O. volvulus* and binds to PCR products of 150-bp length and longer

**Table 2** Comparison of *O. volvulus* mf detection in skin snips using PCR-based DNA detection with subsequent DNA probing and overnight incubation in isotonic saline solution. The biopsies were obtained from 227 infected persons who lived in an area in which annual ivermectin mass treatment had been conducted for 3 years

| PCR | Saline incubation | | | | | |
|---|---|---|---|---|---|---|
| | Mf-positive | | Mf-negative | | Total | |
| | *n* | % | *n* | % | *n* | % |
| Positive | 157 | 69.2 | 16 | 18.9 | 173 | 76.2 |
| Negative | 11 | 4.8 | 43 | 7.1 | 54 | 23.8 |
| Total | 168 | 74.0 | 59 | 26.0 | 227 | 100.0 |

**Table 3** Comparison of *O. volvulus* mf detection in skin snips using PCR-based DNA detection with subsequent DNA probing and collagenase digestion. The biopsies were the same as those described in Table 2

| PCR | Collagenase digestion | | | | | |
|---|---|---|---|---|---|---|
| | Mf-positive | | Mf-negative | | Total | |
| | *n* | % | *n* | % | *n* | % |
| Positive | 163 | 71.8 | 10 | 4.4 | 173 | 76.2 |
| Negative | 16 | 7.1 | 38 | 16.7 | 54 | 23.8 |
| Total | 179 | 78.9 | 48 | 21.1 | 227 | 100.0 |

that were PCR-positive in the first biopsy but mf-negative as determined by collagenase digestion of the second biopsy. The mf densities of the 16 persons who were PCR-negative in one biopsy but mf-positive in the other biopsy ranged from 0.2 to 1.8 mf/mg skin (median 0.5 mf/mg skin). The total number of mf detected in these 16 biopsies ranged from 1 to 8 mf (median 2 mf/skin snip). Since two biopsies were involved in the analysis, we estimated the likelihood for a second biopsy to be mf-negative in relation to the mf content of the first biopsy. The estimated data were based on the results of previous analysis of a 141 pairs of skin snips examined in western Uganda (Fischer et al. 1993) and were compared with those obtained in the 16 persons who were PCR-negative in one biopsy but mf-positive in the other biopsy. Figure 3 shows a very similar trend in the two groups, indicating that the sensitivity of the PCR-based assay is dependent on the mf density. A sensitivity of 83.3% was achieved when the results of the two biopsies, one digested by collagenase and one examined by PCR, were combined.

As a control, skin biopsies from 19 mf-negative persons were tested by PCR. These persons showed nega-

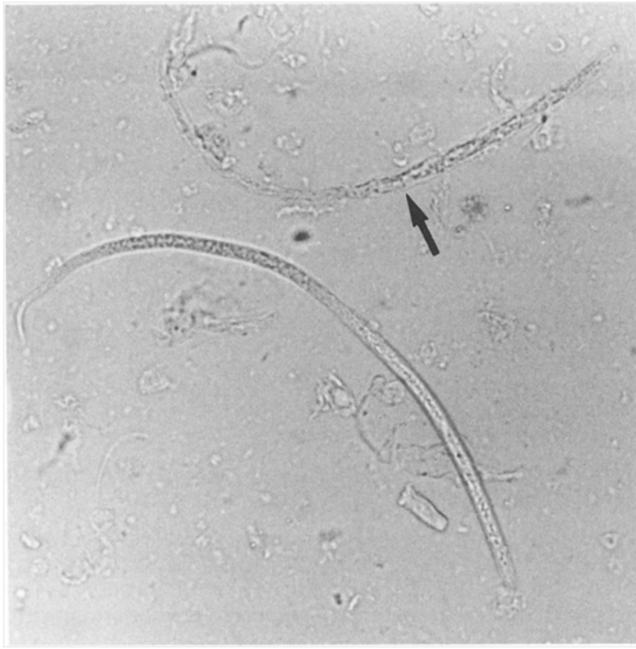

**Fig. 2** Degenerating (*arrow*) and intact microfilariae of *O. volvulus* as detected by the collagenase digestion method. ×320

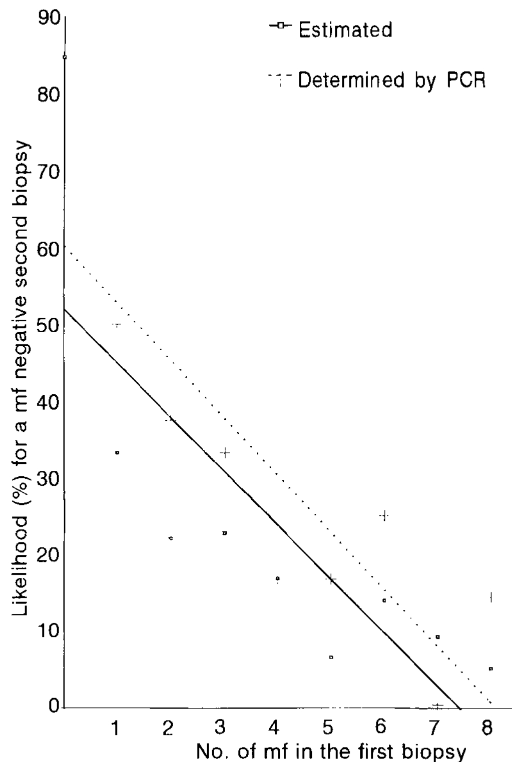


**Fig. 3** Likelihood for a microfilariae (*mf*)-negative second biopsy in dependence on the number of mf in the first biopsy as estimated by parasitological methods or determined by PCR. For further details, see results

tive skin snips in the baseline survey in 1991 as well as during the reexamination. All the samples were negative in the PCR-based assay. Since no false-positive reactions occurred in this group, an assay specificity of more than 95% can be concluded.

## Discussion

The PCR was successfully employed both for the amplification and, in combination with hybridization experiments, for the specification of *Onchocerca volvulus* DNA from various African areas. The general applicability of this assay for the diagnosis of *O. volvulus* infections in Africa can be concluded. The PCR-based detection of *O. volvulus* DNA in skin biopsies of patients from western Uganda showed that the concentration of amplification products was sometimes very low, and hybridization to a sensitive DNA probe was necessary to detect the small amounts of amplified DNA. This indicates that in these samples an inhibition of amplification had probably occurred. Zimmerman and co-workers (1994) also observed this phenomenon. However, the *O. volvulus*-specific DNA probes S9 and OvS2 were very sensitive and suitable for determination of the presence of O-150 of *O. volvulus* from western Uganda.

One aim of the further development of PCR methodologies is to adapt the PCR and DNA hybridization to field conditions. Barker (1994) mentioned the promising results obtained in the Onchocerciasis Control Programme in West Africa using a PCR-based detection of *O. volvulus* in blackflies and subspecific differentiation. In the present study, nonradioactive, DIG-labeled *O. volvulus*-specific DNA oligonucleotides were used for species confirmation. Such DNA probes can easily be obtained by customer synthesis by commercial suppliers. No radioactivity or molecular cloning is necessary to produce the DNA probes, and the assay can be conducted in most parasitology laboratories. The required technical equipment can also be used for PCR detection of other pathogens, and it can be assumed that in the future, such equipment will be a standard prerequisite for a diagnostic laboratory. To minimize the equipment required, further research on the development of a nested PCR for species confirmation is needed.

Is has been reported that in infected persons, *O. volvulus* DNA is present in the skin even if no mf are detectable parasitologically (Freedman et al. 1994). This contrasts with our finding that the PCR-based assay of skin snips has the same sensitivity as the parasitological assessment of mf in skin snips using collagenase digestion. It cannot be excluded that the PCR assay described by Zimmerman et al. (1994) and Elson et al. (1994) may have a higher sensitivity that the one described in the present study, and *O. volvulus* infections can be detected even in the absence of mf in the skin. These authors used a reference method different from ours, they worked on patients' material from Latin America, the biopsies were preserved in an EDTA solution in liquid nitrogen, and

the primers they used were slightly different from ours. The introduction of the enzyme-linked immunosorbent assay (ELISA)/biotin using biotin labeled primers instead of the Southern-blot assay may also increase the sensitivity (Nutman et al. 1994).

However, it appears unlikely that free *O. volvulus* DNA exists in the skin when mf are absent. Dead mf are attacked by enzymes of eosinophils (Wildenburg et al. 1994, 1995), neutrophils (Gallin et al. 1995), and macrophages, and fragments are taken up by phagocytic cells such as multinuclear giant cells and macrophages (Büttner et al. 1988). Light microscopical, electron microscopical, and immunohistological observations reveal that mf disappear within a maximal period of 3 days after microfilaricidal treatment (Büttner et al. 1991; Darge et al. 1991). The fate of DNA of another pathogen, the spirochete *Borrelia burgdorferi*, was recently studied in a mouse model; by at least 3 weeks after successful chemotherapy, no bacteria DNA was detectable in the skin by PCR (Malawista et al. 1994). This finding is in accordance with the hypothesis that *O. volvulus* DNA in the skin does not remain stable for a long period and supports our observation that individuals with proven infection but with no mf in their skin due to ivermectin treatment, which is microfilaricidal but does not kill the adult worms, are in general PCR-negative. It is doubtful that DNA of disintegrating adult *O. volvulus* occurs in the vicinity of onchocercomata, which can be detected by PCR. However, our results also have implications for the diagnosis of onchocerciasis in prepatent infections: it can be assumed that these infections cannot be determined by a PCR-based assay of skin snips, since mf are absent by definition.

Some resports have pointed out that the more skin snips are taken, the more accurate the diagnosis of onchocerciasis using an overnight incubation in RPMI medium (Taylor et al. 1987, 1989). This is true for all techniques involving skin biopsies for the diagnosis of onchocerciasis. However, because skin snipping is an invasive procedure and due to the dangers of viral transmission, the number of skin snips taken should be kept at a minimum. Previously, we reported that after overnight incubation in isotonic solution, about one-third of the mf remained in the skin snip as determined using collagenase digestion as the reference method (Fischer et al. 1993). Zimmerman et al. (1994) showed that 13 of 34 persons who were mf-negative in 2 skin snips after a 24-h incubation in isotonic saline were positive in the PCR assay performed on these biopsies. This finding supports our previous report and indirectly indicates a similar sensitivity for collagenase digestion and the PCR assay. In hyperendemic onchocerciasis foci with high mf densities, only two-thirds of all mf need to emerge from a biopsy for a representative result. In other regions it could be necessary to apply the collagenase digestion or PCR method. The relatively high cost of the PCR equipment as well as the cost of each reaction may limit application of the PCR for diagnosis, especially in African laboratories.

In hypoendemic areas a topical Mazzotti test in combination with the skin snip for the exclusion of a patent *O. volvulus* infection has been recommended (Kilian 1988). Although the sensitivity of this test is also dependent on the mf density of the patient, it is noninvasive and the probability of inducing a reaction can be increased by the application of a DEC lotion on an area of skin larger than that usually taken for a biopsy.

In conclusion, this PCR-based assay can be used for amplification and for specification of *O. volvulus* DNA using DIG-labeled DNA probes from various African countries and parasite strains. The sensitivity of the collagenase digestion method and the PCR assay applied on skin biopsies can be increased only by the use of larger or more numerous biopsies. Since no free DNA is generally present in skin biopsies, only DNA of mf or fragments of mf is detectable by PCR. The presence of parasites can also be more easily and more cheaply proven by collagenase digestion of skin snips. However, if a strain differentiation is needed and adult worms are not available, the PCR-based amplification of DNA and the use of strain-specific probes are required.

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